

INVESTIGATION OF THE BIOLOGIC ACTIVITY AND STRUCTURE  
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R L HUNTER ET AL. 15 JUL 87 N00014-86-K-0456

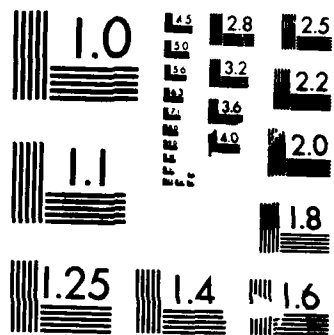
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|---|-------|--|---|--|---------------------------|
| 1a RE<br>(1)  |       |  | 1b RESTRICTIVE MARKINGS<br>NA   |  |                           |
| 2a SE<br>NA   |       |  | 3. DISTRIBUTION/AVAILABILITY OF REPORT<br>Distribution Unlimited                                    |  |                           |
| 2b DECLASSIFICATION/DOWNGRADING SCHEDULE<br>NA  |       |  | 5. MONITORING ORGANIZATION REPORT NUMBER(S)<br>NA   |  |                           |
| 4 PERFORMING ORGANIZATION REPORT NUMBER(S)<br>Emory University  |       |  | 7a NAME OF MONITORING ORGANIZATION<br>Office of Naval Research                                      |  |                           |
| 6a NAME OF PERFORMING ORGANIZATION<br>Emory University  |       | 6b OFFICE SYMBOL<br>(if applicable)<br>NA                          | 7b ADDRESS (City, State, and ZIP Code)<br>800 North Quincy Street<br>Arlington, Virginia 22217-5000 |  |                           |
| 6c ADDRESS (City, State, and ZIP Code)<br>1364 Clifton Road<br>Atlanta, Georgia 30322   |       | 9 PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER<br>N00014-86-K-0456 |   |  |                           |
| 8a NAME OF FUNDING/SPONSORING ORGANIZATION<br>Office of Naval Research  |       | 8b OFFICE SYMBOL<br>(if applicable)<br>ONR                         | 10 SOURCE OF FUNDING NUMBERS  |  |                           |
| 8c ADDRESS (City, State, and ZIP Code)<br>800 North Quincy Street<br>Arlington, Virginia 22217-5000   |       | PROGRAM<br>ELEMENT NO<br>61153N                                    | PROJECT<br>NO<br>RR04108  | TASK<br>NO   | WORK UNIT<br>ACCESSION NO |
| 11 TITLE (Include Security Classification) (U) Investigation of the biologic activity and structure function relationships of synthetic polymers which act as hormones, behavior modifiers and thymic immunomodulators.   |       |  |   |  |                           |
| 12 PERSONAL AUTHOR(S)<br>Hunter, Robert Lee, Check, Irene Jocius, Neill, Darryl Boyd  |       |  |   |  |                           |
| 13a TYPE OF REPORT<br>Annual  |       | 13b TIME COVERED<br>FROM 7/15/86 TO 7/15/87                        |   | 14 DATE OF REPORT (Year, Month, Day)<br>1987 July 15 |                           |
| 15 PAGE COUNT   |       |  |   |  |                           |
| 16 SUPPLEMENTARY NOTATION   |       |  |   |  |                           |
| 17 COSATI CODES   |       |  | 18 SUBJECT TERMS (Continue on reverse if necessary and identify by block number)                    |  |                           |
| FIELD   | GROUP | SUB-GROUP  | thymus, stress, neuropeptide analogues, ionophore,  |  |                           |
| 08  |       |  | central nervous system, behavior modification, Polyphore,   |  |                           |
|   |       |  | block copolymer   |  |                           |
| 19 ABSTRACT (Continue on reverse if necessary and identify by block number)   |       |  |   |  |                           |
| This project attempts to probe interrelationships between the central nervous, immune and endocrine systems. The key departure was the discovery that certain synthetic copolymers stimulate hyperplasia of cortical lymphocytes in the thymus and increased motor behavior. These copolymers, polyphores, are monovalent cation-selective ionophores. A variety of investigations suggested that these copolymers might mimic the activity of neuropeptides either by interacting with their receptors or by modifying cation fluxes across cell membranes. The experiments in this project have been designed to characterize the effects of polyphores more completely and to look for changes which might be attributed to the action of neurotransmitter analogues. Retrospective analysis of the first year's data demonstrated stimulatory effects of the polyphore copolymer on the |       |  |   |  |                           |
| 20 DISTRIBUTION/AVAILABILITY OF ABSTRACT<br><input checked="" type="checkbox"/> UNCLASSIFIED/UNLIMITED <input type="checkbox"/> SAME AS RPT <input type="checkbox"/> DTIC USERS   |       |  | 21 ABSTRACT SECURITY CLASSIFICATION<br>(U)  |  |                           |
| 22a NAME OF RESPONSIBLE INDIVIDUAL<br>Dr. J.A. Majde  |       |  | 22b TELEPHONE (Include Area Code)<br>202/696-4055   |  | 22c OFFICE SYMBOL<br>ONR  |

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Block 19. Abstract, continued

✓ The thymus and on motor activity primarily in animals which had undergone severe stress. Consequently, ~~our~~ current working hypothesis is that the ionophore copolymers somehow modify the reaction to stressful stimuli. This modification includes an increase in motor activity, a reduction in involution of the thymus and a stimulation of proliferation of cells in the cortex of thymus. During the coming year, animals will be treated with stressful conditions in a controlled fashion in an effort to test the hypothesis that the copolymer modifies the stress reaction. Several stressful stimuli, including food and water deprivation, immobilization and rewarding brain stimuli, will be evaluated. In addition, stress simulating a chronic infectious disease (the intravenous injection of a BCG cell wall emulsion which produces longlasting pulmonary granulomas) will be used. Assays year will include hormonal measurements in addition to the behavioral, gross and microscopic pathologic studies similar to those done this year.

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## Annual Report

**Investigation of the biologic activity and structure function relationships of synthetic polymers which act as hormones, behavior modifiers and thymic immunomodulators.**

**O.N.R. Contract #N00014-86-K-0456**

### Introduction

In the course of investigating synthetic immunomodulating agents, we found that a particular alkoxyated amine copolymer, 'Polyphore 32:5', produced a rapid and sustained increase in the size of the thymus in rats and mice. This increase was due to cortical T lymphocytes. T lymphoid cell numbers in peripheral lymphoid tissues were also increased. Injection of polyphore produced a number of other effects including increased motor activity and diuresis which suggested that they affected the central nervous system in addition to the thymus. Studies in vitro demonstrated that polyphores were ionophores with selectivity for monovalent cations and that they were potent stimulators of histamine release from mast cells. Finally, certain structural and physicochemical properties suggested to us that polyphores might be analogues of substance P or somatostatin. Molecules which affect sodium gradients across cell membranes and mimic the activity of neuropeptides might be expected to have diverse effects on excitable cell systems. This project was designed to characterize the effects of polyphore copolymers on the thymus and other elements of the immune system and to evaluate selected behavioral effects.

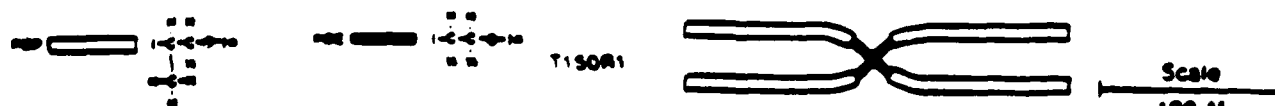
**Chemical structure and nomenclature of polyphore copolymers:** The compounds used in these studies are nonionic block copolymer surfactants which consist of a central initiator of ethylenediamine linked to blocks of hydrophilic polyoxyethylene (POE) flanked by hydrophobic blocks of polyoxypropylene (POP) as shown in Figure 1. They comprise a series of chemically synthesized copolymers which vary in length, relative proportions of POP and POE and molecular configuration. The copolymers, designated polyphores, are manufactured by BASF as Reverse Tetronic Polyols or reverse octoblock copolymers. Some are also available from CytRx Corporation, Atlanta, Georgia. The studies in this project have concentrated on Reverse Tetronic Polyol T150R1 which is also known as Polyphore 32:5. Each of the four chains of POE have an average of five ethylene oxide moieties which each of the POP chain have an average of 32 moieties.

**Ionophore Activity:** The activity of the polyphores as ionophores has been demonstrated in several systems. First, using artificial lipid membranes, we demonstrated that the polyphores were able to transport sodium, potassium and hydrogen across an artificial lipid membrane in a voltage dependent fashion with kinetics which suggested a carrier rather than a channel-forming mechanism. Secondly, using red blood cells and radioactive cation isotopes, we demonstrated that the polyphores could mediate the exchange of external sodium with potassium inside of red blood cells. In

this system, the polyphores had approximately equal affinity for sodium and potassium, but no demonstrable affinity for calcium.

Figure 1. Structures and commercial names of the copolymers.

| <u>BASF name</u> | <u>CytRX name</u> | <u>M.W.</u> | <u>Structure</u>   |
|------------------|-------------------|-------------|--|
| T110R1           | Polyphore 21:3    | 5520        | $\begin{matrix} 21 & 3 & 3 & 21 \\ 21 & 3 & 3 & 21 \end{matrix}$     |
| T130R1           | Polyphore 27:4    | 6800        | $\begin{matrix} 27 & 4 & 4 & 27 \\ 27 & 4 & 4 & 27 \end{matrix}$     |
| T130R2           | Polyphore 27:10   | 7740        | $\begin{matrix} 27 & 10 & 10 & 27 \\ 27 & 10 & 10 & 27 \end{matrix}$ |
| T150R1           | Polyphore 32:5    | 8000        | $\begin{matrix} 32 & 5 & 5 & 32 \\ 32 & 5 & 5 & 32 \end{matrix}$     |
| T150R4           | Polyphore 32:26   | 11800       | $\begin{matrix} 32 & 26 & 26 & 32 \\ 32 & 26 & 26 & 32 \end{matrix}$ |
| Tetronic T1501   |                   | 8000        | $\begin{matrix} 5 & 32 & 32 & 5 \\ 5 & 32 & 32 & 5 \end{matrix}$     |



The copolymers are composed of blocks of polyoxyethylene (POE) and polyoxypropylene (POP) attached to a central ethylene-diamine group. The numbers in the structure column represent the average number of groups of oxyethylene and oxypropylene (underlined) in each block rounded to the nearest integer. The diagram is drawn to scale showing the four chain structure of the copolymers.

Extensive studies were done evaluating the ability of polyphores to produce histamine release from murine peritoneal mast cells and human peripheral blood basophils. The polyphores produced an energy dependent active release of histamine from both types of cells. The polyphores acted synergistically with anti IgE, concanavalin A and phorbol esters, but not with the calcium ionophore A23187. Even though the polyphores had no demonstrable affinity for calcium, calcium in the medium was required for release of histamine. Each of the hydrophobic copolymers tested had qualitatively similar activities as ionophores, but they differed in intensity. Polyphore 27:10 (T130R2) was the most effective ionophore. Polyphore 32:5 (T150R1) is much less toxic and in preliminary studies demonstrated marked stimulatory effects on cortical thymocytes *in vivo*. We proposed that the ionophores caused an increase of intracellular sodium which was exchanged for calcium and that an increased level of intracellular calcium was responsible for the increased excitability of these cells. This mechanism has potential activity in many types of cells.

**Effects on the thymus:** In the course of evaluating polyphores as vaccine adjuvants, we found that injections of Polyphore 32:5 caused thymic hyperplasia in mice. In one experiment, groups of six-week-old female ICR outbred mice were injected in the rear footpads with an oil and water emulsion containing 2.5 mg of Polyphore 32:5. Animals were killed at intervals and their thymus glands weighed. Microscopic examination revealed that the changes in the thymus were limited almost exclusively to cortical thymocytes (Figure 2). Their numbers were markedly reduced at

**Figure 2. Weight and histologic changes in the thymus.**

| <u>Weight of thymus in milligrams</u> |                |                |
|---------------------------------------|----------------|----------------|
| <u>Agent</u>                          | <u>2 weeks</u> | <u>6 weeks</u> |
| Polyphore 32:5                        | 132 +/- 13     | 90 +/- 7       |
| Vehicle control                       | 79 +/- 5       | 37 +/- 8       |



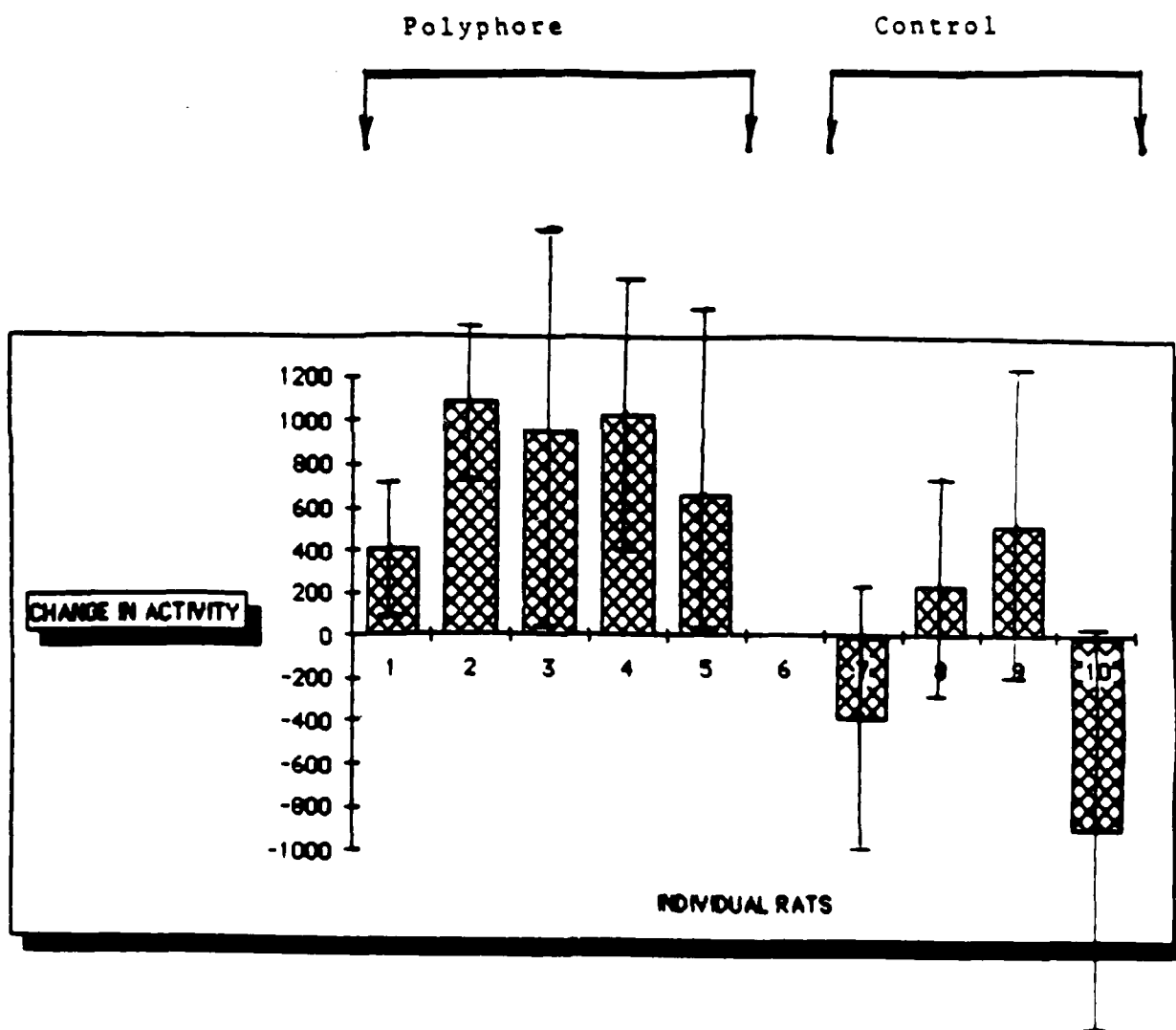
three days after injection but approached normal by one week. After that time, the size of the glands of treated animals increased over those of controls. The medullary areas demonstrated little change, but the cortical areas were much larger than normal. They were composed of large and small lymphoid cells. The effects proved to be very long lasting. Animals sacrificed six to ten months after a single injection still demonstrated hypertrophy of the cortical lymphoid elements as compared with matched controls. In the course of these experiments, we noticed that treated animals appeared to be more active in their cages than untreated ones, especially when handled. Consequently, we initiated a study to quantitate motor activity.

**Effects on motor activity:** Nine rats were acclimatized with some difficulty to our animal quarters. Baseline locomotor activity was determined on each animal three days a week for two weeks using an Omnitech activity measuring device which uses infrared light beams to measure

movement of the animal. After collecting baseline data, five of the animals were injected subcutaneously with 5.0 mg of Polyphore 32:5. The other four were injected with vehicle as controls. Each animal's activity was measured for an additional two weeks. The data were analyzed as paired observations subtracting the pretreatment from posttreatment activity scores for each animal.

The activity increased in four of the five treated animals but in none of the controls (Figure 3). The composite change in activity for the group of treated animals showed a highly statistically significant increase as compared with controls.

**Figure 3. Change in activity in Sprague-Dawley rats induced by Polyphore 32:5.**



The change in activity in individual rats brought about by injections of Polyphore 32:5 compared with controls. The vertical axis shows the mean difference in the number of times animals broke a light beam during a 16 minute test in the period after injection. Four out of five of the injected animals showed statistically significant values by the T tests at the  $P < 0.05$  level. None of the controls demonstrated significant changes.



### **Specific Aims for Year 1.**

1. Optimize the dose and time parameters for production of thymic hyperplasia and behavioral changes by Polyphore 32:5 in Lewis rats.
2. Immunochemically and functionally characterize the effects of Polyphore 32:5 on thymus and peripheral lymphoid tissue.
3. Investigate hormonal mechanisms which might mediate the effects of Polyphore 32:5.

### **Progress Report.**

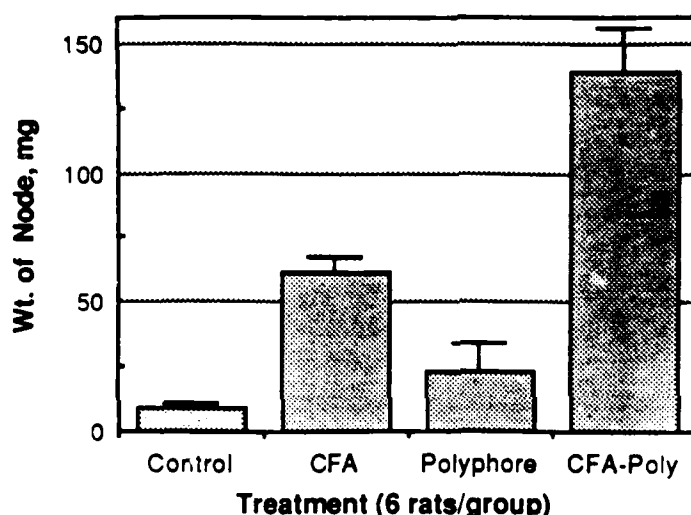
**Summary:** A number of variables including the species and strain of animal, dose and route of administration of polyphore were evaluated for their effects on immunological and behavioral measurements. It became evident that additional variables related to stress confounded the interpretation of the results. These included the location of the cages (temperature, light), food and/or water deprivation, single vs. group housing, footpad inflammation, co-existing infections, etc. We observed that the most positive effects of polyphores had been observed in animals who had undergone severe stress. The positive results could not be attributed to random variation or noise in the system. When present, they were quite clear and distinct from controls. Parallel experiments with adrenalectomized and hypophysectomized mice suggested that the effects required intact adrenal glands, but not the hypophysis. Consequently, we have formed a modified hypothesis to be evaluated during the coming year; namely that the polyphores act to modulate the effects of stress.

**Experiments with rats:** The first experiment was designed to evaluate multiple parameters of rats in metabolism cages for use as a baseline for further immunologic, endocrinologic and behavioral studies. Male Sprague-Dawley rats were acclimatized to metabolism cages in a new animal facility. They were then injected with 2.5 mg of Polyphore 32:5 in the rear footpad. Food and water intake, urine output, body weight and blood samples were measured serially. Animals were sacrificed for tissue studies at 21 days. No significant effects could be attributed to treatment with copolymer. However, this experiment revealed that animals housed on the top shelves of a rack of metabolism cages where the room air is warmer and there is more light react differently than those housed on the bottom shelves, and gave us considerable experience in maintaining animals in metabolism cages. It also made us aware of the potential complications of the stress of single-housing and foot-pad inflammation on our results. We decided to study an inbred strain of rats to further decrease individual variation.

Thus, a second experiment compared single vs. group housed Lewis rats in standard cages, and tested footpad vs. neck injections in a similar protocol. This demonstrated a debilitating effect of footpad injections in that these animals drank less water for up to two weeks following injection. However, this group also showed a transient increase in water intake immediately post-injection. We noted that these animals seemed stressed by the injection. They may have arrived from the supplier being ill, or been stressed in transit. Differences were also observed between single and group-housed animals.

A large experiment using 70 Lewis rats was then undertaken to evaluate several of the variables (injection route, housing conditions, vehicle) in a controlled fashion. It again demonstrated decreased water consumption in animals who had received footpad injections. Rats receiving subcutaneous injections in the scruff of the neck did not show decreased water intake. Because of this, we have subsequently used only neck injections for animals intended for behavioral studies. The results did not demonstrate any increase in the weight of thymuses over those of controls. However, both the spleen and popliteal lymph nodes demonstrated sustained hyperplasia in animals which had received a combination of Polyphore 32:5 with Freund's complete adjuvant (CFA). Such changes were not seen with either material injected alone in group-housed rats (Figure 4). The animals used in these experiments were all healthy throughout.

**Figure 4. Effect of Polyphore 32:5 and CFA on rat popliteal lymph nodes.**

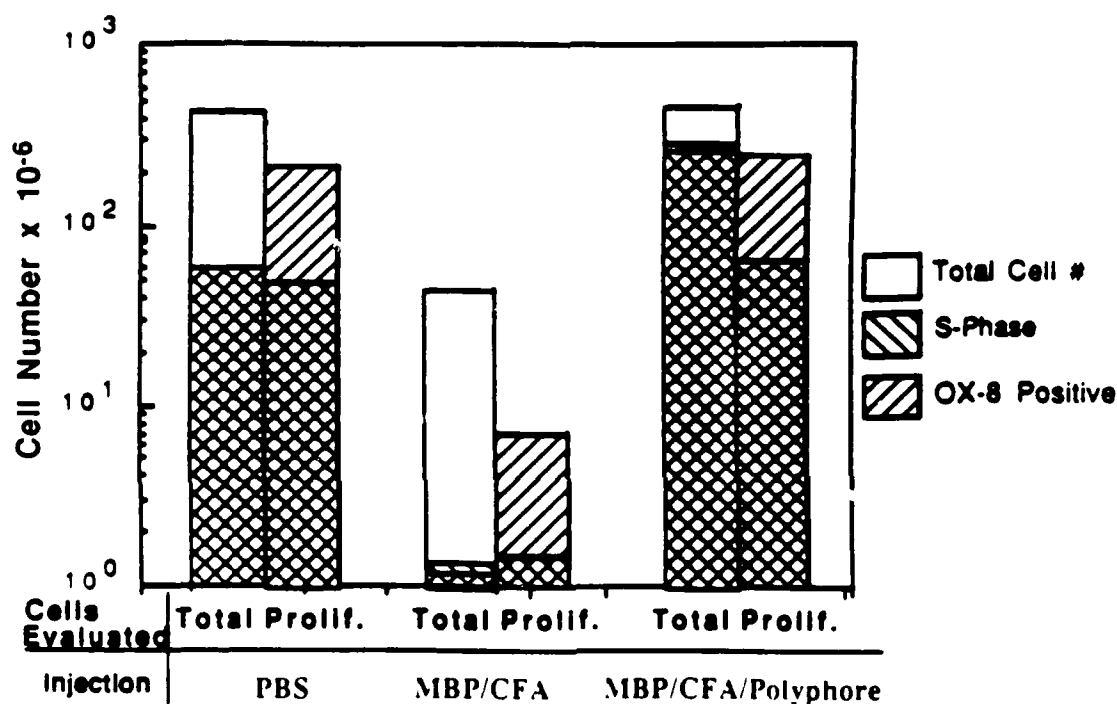


Group-housed Lewis rats (6/group) were injected in the footpads with Polyphore 32:5 (2.5 mg) with or without complete Freund's adjuvant (CFA). The controls included uninjected animals and animals injected with CFA alone. They were sacrificed on day 41 and organs were removed and weighed.

A graduate student project carried out concurrently with the present project produced results supporting the hypothesis that the effects of polyphore treatment on the thymus are most evident in conditions of deleterious stress. In these experiments, the stress was that of paralysis due to experimental allergic encephalomyelitis (EAE) induced in Lewis rats by injections of myelin basic protein (MBP) in complete Freund's adjuvant (CFA). It was found that injections of Polyphore 32:5 effectively suppressed the induction of EAE even when given several days after the encephalitogenic injection. On day 13 during the peak of the disease, thymuses of animals with EAE weighed an average of only 159 mg, contained a small number of cells, and only 4% of the cells were in the DNA synthesis (S) phase of the cell cycle as assessed by FACS analysis. Thymuses of

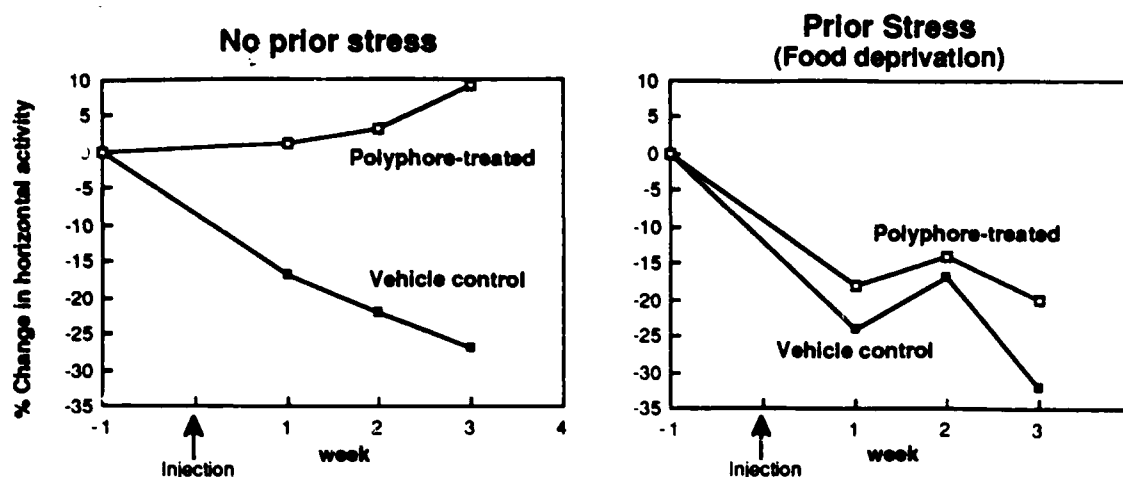
animals whose EAE had been suppressed weighed an average of 252 mg. had a greater cell number, and 60% of the cells were in S phase. They were predominantly of the suppressor (Ox 8) phenotype. When compared with phosphate buffered saline (PBS) controls, however, the cell numbers in polyphore-treated animals were similar and there was only a small increase in the number of proliferating cells (Figure 5). This was additional evidence that polyphores may modulate the effects of another noxious stimulus, in this case the induction of EAE, on thymus size.

**Figure 5. Effects on MBP/CFA with or without Polyphore 32:5 on thymus cell populations 13 days after injection.**



The most recent experiment conducted in rats studied the effects of polyphore in Lewis rats stressed by food deprivation for five days. Activity was measured during a 14-day period of acclimatization. 5 mg of copolymer were injected in the neck in an oil and water vehicle, and activity measurements were continued. The untreated animals showed a progressive decrease in activity as they became further acclimatized to their cages. Although this experiment is now in progress, it appears that the treated rats are not showing the same decline in activity (Figure 6). An additional animal was inadvertently deprived of water for several days because of a blocked water bottle. This animal demonstrated a very high level of activity throughout the experiment. Further experiments in the coming year under controlled types and degrees of stress are planned to evaluate the idea that Polyphore 32:5 may decrease the deleterious effects of stress.

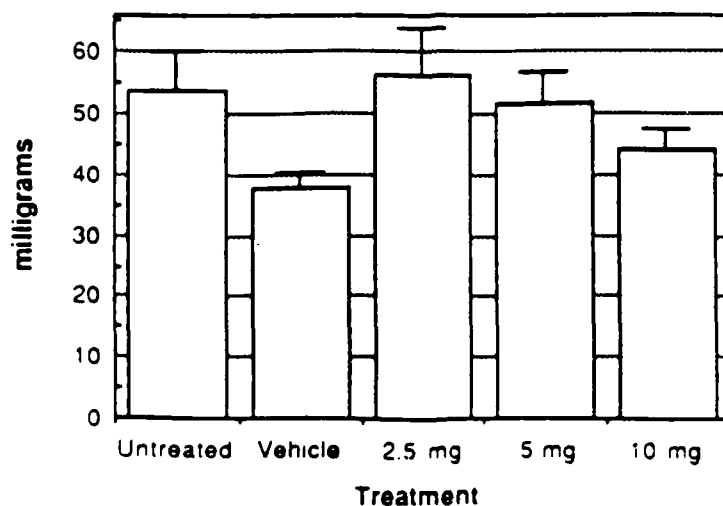
**Figure 6. Effects of Polyphore 32:5 on spontaneous activity in rats.**



Effects of polyphore 32:5 on spontaneous activity in rats previously stressed by food deprivation or non-stressed. The Polyphore appears to increase activity in non-food-deprived animals and to ameliorate the reduction in activity from the stress of food-deprivation or vehicle injection. Following food deprivation or control treatment and baseline activity measurements as described in the text. Polyphore (5 rats) or vehicle (4 rats) was injected at week 0. The mean percent change in activity is expressed relative to the values at week -1.

**Experiments with mice:** We conducted an experiment in ICR outbred mice which supported the concept that effects of polyphore on the thymus involve immunomodulating the deleterious stressful effects of other agents, in this

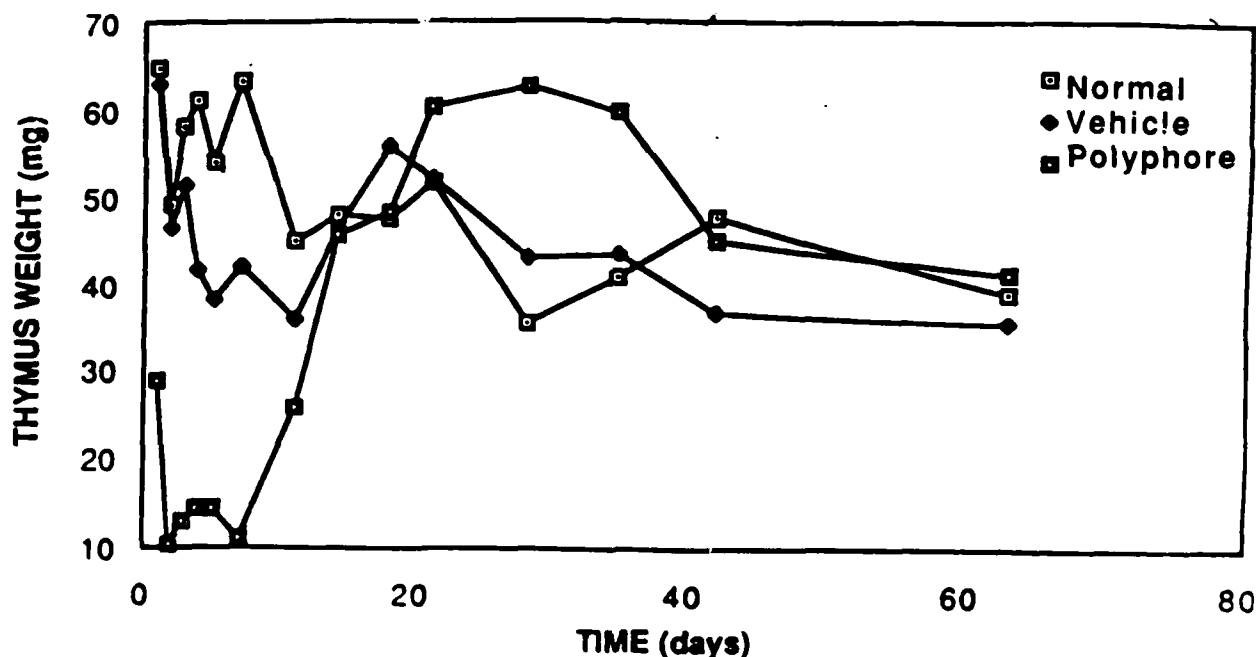
**Figure 7. Effects of Polyphore 32:5 on thymus weight of ICR mice on day 31.**



case, that of the injection vehicle. Animals were injected with 2.5, 5 or 10 mg of polyphore in a vehicle consisting of an oil and water emulsion of Drakeol or with the vehicle itself. After 31 days, the thymuses of the animals injected with the vehicle were significantly smaller than those of any other groups. The copolymer treated groups had thymuses near the size of the untreated controls which decreased somewhat with increasing dose (Figure 7).

Additional related studies were carried out by a graduate student on the effects of copolymers in mice. Groups of female balb/c mice were injected with 2.5 mg of Polyphore 32:5 in their footpads at seven to eight weeks of age. The sizes of their spleens and thymuses were determined at intervals thereafter (Figure 8a and 8b). The thymuses initially decreased in weight but returned to normal in approximately two weeks and were significantly larger than those of controls during weeks three to six. The size of the spleens increased dramatically over those of controls after the first week and persisted for six weeks.

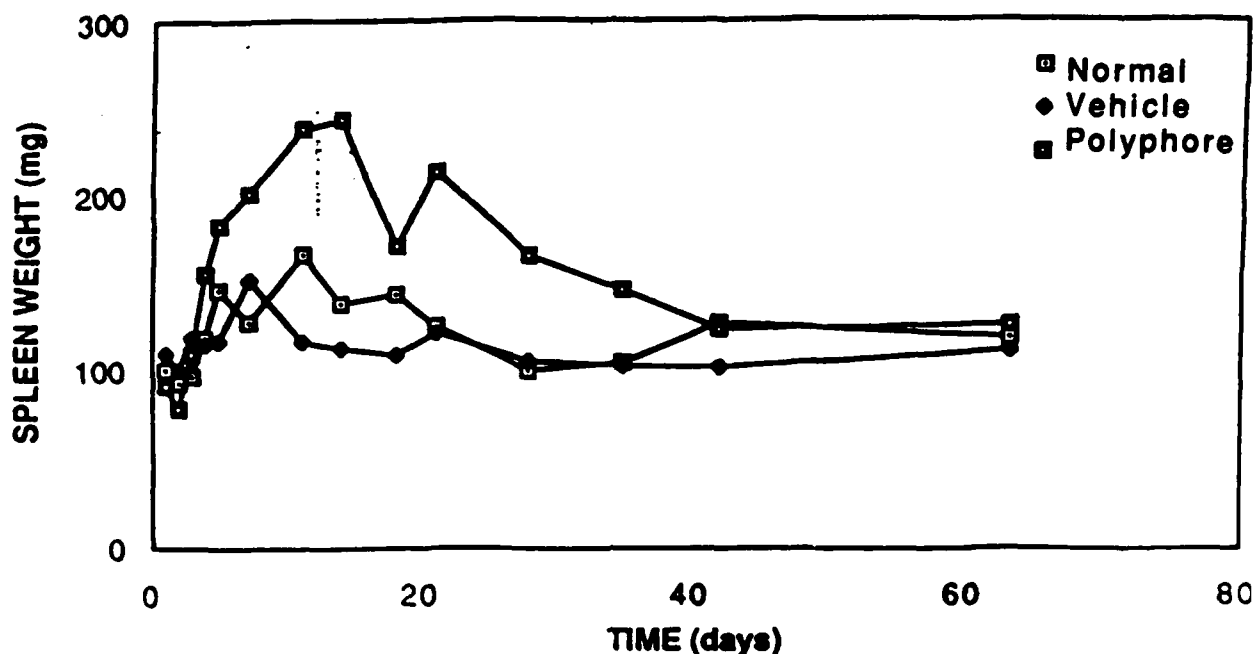
Figure 8A. Footpad injection of Polyphore 32:5 in balb/c mice: Time course of changes in thymus weight.



\*n=3 for each time point.

\*A total of 2.5 mg Polyphore 32:5 (oil in water emulsion) was injected in the footpads of 7-8 week old female balb/c mice. Vehicle injected mice received the emulsion with no copolymer.

**Figure 8B. Footpad injection of Polyphore 32:5 in balb/c mice: Time course of changes in spleen weight.**

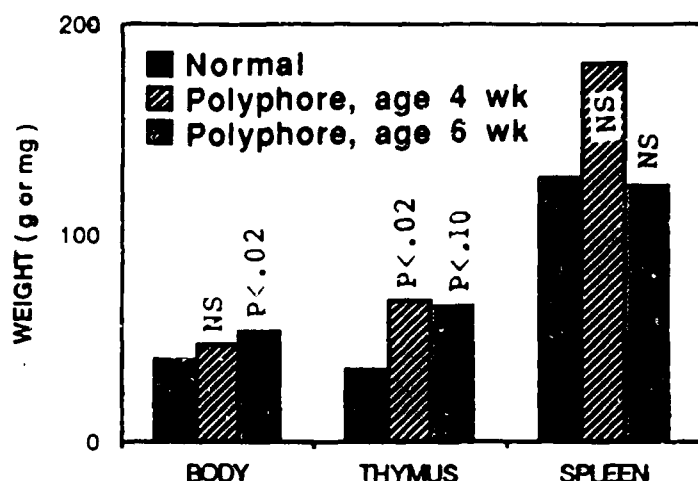


\*n=3 for each time point.

\*A total of 2.5 mg Polyphore 32:5 (oil in water emulsion) was injected in the footpads of 7-8 week old balb/c mice. Vehicle injected mice received the emulsion with no polyphore 32:5.

A large group of female ICR mice had been injected with 2.5 mg of Polyphore 32:5 in an oil and water emulsion as part of earlier studies. Animals sacrificed at various times from six to twelve months had demonstrated small but significant increases in their thymus weight. Figure 9 shows the results from animals sacrificed at 18 months following a single injection. Their thymuses were significantly enlarged in animals which had been injected at either four weeks and six weeks of age. Histologically, there were many more lymphoid cells in the cortices of these animals than in the uninjected controls. These results further confirmed our previous observation that the copolymer produced long term effects consistently even though the short term effects were difficult to reproduce.

Figure 9. Long term effects of Polyphore 32:5 on mice.



Mice (3/group) were injected in the footpads with 2.5 mg Polyphore 32:5 (oil in water emulsion) at either 4 weeks or 6 weeks of age. All mice were sacrificed 18 months after injections and compared to injected controls.

Additional studies were done with adrenalectomized or hypophysectomized animals. Female balb/c mice six to eight weeks of age which had been adrenalectomized were acclimatized to their housing and injected with 2.5 mg of Polyphore 32:5 in an oil and water emulsion. The thymuses of the controls demonstrated a reduction in size followed by a rebound towards normal at two weeks. Adrenalectomy largely abolished this reduction in thymus size. Histologic examination revealed that the adrenalectomies were not complete. We believe that this supports the hypothesis that the early involution of the thymus following polyphore injection is dependent upon the adrenal. Studies with hypophysectomized animals demonstrated different results. The hypophysectomy was unable to reduce involution of the thymus produced by the polyphore. These animals however did demonstrate a reduction in adrenal weight at three days followed by an increase at seven days. This suggests the release of cortical steroids followed by a rebound hyperplasia. These data are interpreted to suggest that the pituitary is being bypassed by the copolymer and that its effect is mediated in part via the adrenals. This is consistent with the hypothesis that an ACTH-like substance produced by other cells, perhaps splenic lymphocytes or macrophages, is responsible. We are particularly intrigued by the possibility that this might be related to the induction of interferon, since there is evidence from other systems that the polyphore copolymers might serve as interferon inducers.

In summary, the experiments carried out with healthy animals housed under optimum conditions failed to show significant effects on behavior, thymus, blood chemistries or other parameters which we measured. However, animals which were either infected, starved or housed under suboptimal conditions frequently showed significant effects. The mice which demonstrated thymic effects lasting for a year and a half were obtained at a

time when our colonies were infected with hepatitis. The first experiments with rats which demonstrated the most significant changes in activity used animals which we acquired during a time of transition in our animal care facility. They arrived in such poor condition that we watched them for a week to be sure they would survive before initiating an experiment. These studies together with a number of other observations and the results of the studies with adrenalectomies and hypophysectomy suggested that our hypothesis needs to be modified. Our new working hypothesis is that the ionophore copolymers somehow modify the reaction to stressful stimuli. This modification includes an increase in motor activity, a reduction in involution of the thymus and a stimulation of proliferation of cells in the cortex of thymus.

#### **Specific Aims for the Coming Year:**

Animals will be treated with stressful conditions in a controlled fashion in an effort to test the hypothesis that the copolymer modifies the stress reaction. Several stressful stimuli will be evaluated. Assays during the coming year will include hormonal measurements in addition to the behavioral, gross and microscopic pathologic studies similar to those done this year. Once we have identified the conditions to produce the changes in the immune system and behavior, other studies as outlined in the application will be carried out to evaluate them further.

First, we will characterize the strain of rat which we use with respect to our anatomical, physiological, and behavioral parameters of interest. This is needed because in some of our experiments of the previous year we have had to deal with moving baselines, particularly for thymus weight, which we attribute to the effects of various kinds of stress. The parameters we will utilize will include: thymus weight and histology, spleen weight and histology, blood chemistry, food and water intake, and spontaneous activity. We will collect these data from untreated male Lewis rats at ages 2, 4, and 6 months. These data will provide the baseline which we need to better examine our polyphore copolymer effects.

Most of our efforts will be devoted to evaluating our new working hypothesis that the drug effects are revealed in stressful, not nonstressful, conditions. We will subject Lewis rats to a variety of stressors and collect the above-mentioned measures. Stressors will include food and water deprivation and immobilization. These are commonly used in the "stress" literature. Based on our success with EAE, we will evaluate a stress simulating a chronic infection, the intravenous injection of a BCG cell wall emulsion which produces long-lasting pulmonary granulomas. Finally, an unusual stress, rewarding brain stimulation will be evaluated. This procedure is interesting because the animals respond at very high rates to receive this stimulation, yet the little data available indicate that very high blood levels of hormones typically thought to reflect stress (such as corticosteroids) are produced. Rats will respond to electrically stimulated hypothalamic areas through chronically implanted electrodes for hours a day, so this may be an easy, humane way of producing a stress state. The laboratory of Dr. Neill of our group is equipped to do this testing and he has years of experience with brain stimulation experiments.



After identifying a procedure which in our hands results in reproducible indices of stress, particularly corticosteroid elevation and thymic involution in rats, we will examine the effect of Polyphore 32:5 in rats who have previously experienced stress and rats who are going to be stressed. Finally, because Polyphore 32:5 may increase cell excitability, we are interested in acute effects which may not have been observed in our previous experiments. Pharmacological studies have shown that drugs which produce general neuronal excitation often result in increased behavioral activity and reactivity. We will test acute injections of Polyphore 32:5 in rats previously baselined on measures of general activity (a procedure we use now) and a test of working memory described in our original proposal. The memory test will consist of training rats to alternate responses between two levers when the time between trials is lengthened to the point that performance normally deteriorates. In the activity test, we would expect spontaneous activity to increase; in the memory test, we would expect better memorial performance.

#### **Publications:**

Houssami, R., Check, I.J. and Hunter, R.L. Immunomodulating effects of synthetic block copolymer T150R1 on mouse thymus and spleen. Federation Proceedings 46:543 (1987).

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